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Gas 6 promotes Axl-mediated survival in pulmonary endothelial cells

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Healy, Aileen M., John J. Schwartz, Xiahui Zhu, Brian E. Herrick, Brian Varnum, and Harrison W. Farber. Gas 6 promotes Axl-mediated survival in pulmonary endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 280: L1273–L1281, 2001.—We examined Gas 6-Axl interactions in human pulmonary artery endothelial cells (HPAEC) and in Axl-transduced HPAEC to test Gas 6 function during endothelial cell survival. We identified the 5.0-kb Axl, 4.2-kb Rse, and 2.6-kb Gas 6 mRNAs in HPAEC. Immunoprecipitation and Western blotting confirmed the presence of these proteins. Gas 6 is present in cell-associated and secreted fractions of growth-arrested HPAEC, independent of cell density. In addition, the Axl receptor is constitutively phosphorylated in growth-arrested cultures, and exogenous Gas 6 enhanced Axl phosphorylation threefold. Gas 6 added to growth-arrested HPAEC resulted in a significant increase in cell number (1.5 nM Gas 6 increased cell number 35%). Flow cytometry revealed that Gas 6 treatment resulted in 28% fewer apoptosing cells. Transduction of a full-length Axl cDNA into HPAEC resulted in 54% fewer apoptosing cells after Gas 6 treatment. Collectively, the data demonstrate antiapoptotic activities for Gas 6 in HPAEC and suggest that Gas 6 signaling may be relevant to endothelial cell survival in the quiescent environment of the vessel wall.

Rse; apoptosis; signal transduction

THE QUIESCENT, NONTHROMBOGENIC phenotype of the vascular endothelium is essential to hemostasis. Under normal conditions, endothelial cell turnover in the vessel wall is relatively low compared with other somatic cell types. However, under certain pathological conditions (e.g., atherosclerosis, pulmonary hypertension, and thrombotic thrombocytopenic purpura), endothelial cell proliferation occurs (9, 18, 20). Endothelial proliferation is associated with increased apoptosis, which in turn generates a prothrombotic phenotype (5). Dysregulation of the endothelial cell phenotype implies that endogenous signaling pathways exist to control cell survival and thus maintain hemostasis.

Gas 6, the product of the growth arrest-specific gene 6, is a soluble factor implicated in the regulation of multiple cellular functions, including growth, survival, adhesion, and chemotaxis (2, 10, 12, 30, 31). Gas 6 signaling is transduced via ligation with three known receptor tyrosine kinases (RTK), Axl (also UFO and Ark) (33), Rse (also Sky, Brt, Tyro-3) (19), and Mer (29). In addition, Gas 6 function is cell-type specific. For example, Gas 6-Axl interactions result in mitogenic and antiapoptotic responses in NIH/3T3 fibroblasts and vascular smooth muscle cells (12, 13, 30, 31). However, Gas 6-Axl interactions mediate cellular aggregation in the murine myeloid 32D cells but show none of the mitogenic or survival activities found in other cell types (26).

There is increasing evidence to suggest that Gas 6 regulates important aspects of vessel wall function. In vascular smooth muscle cells grown in culture, Gas 6 is a growth-potentiating factor for the G protein-coupled receptor agonists thrombin and angiotensin II (30). Gas 6 also prevents growth arrest-induced death and promotes chemotaxis in vascular smooth muscle cells (10, 31). Gas 6 is expressed in vascular endothelial cells (24, 37) and inhibits granulocyte adhesion to activated endothelial cells in vitro (2). In human umbilical vein endothelial cells (HUVEC), Gas 6 promotes cellular viability in the absence of growth factors (34). In vivo, balloon catheterization of rat carotid arteries induces Gas 6 expression within the neointima (27), indicating that Gas 6 is positioned to regulate the vascular response to injury.

Recent studies have begun to address the function for each of the Gas 6 receptors. For example, mice containing targeted deletions of any one receptor, Axl, Rse, or Mer, reveal no overt phenotype (22). However, deletion of all three receptors results in viable animals with multiple abnormalities, the most prominent being male sterility, but noted among the various phenotypes was increased apoptosis in the vessel wall (22). Overexpression of the Axl receptor in cells of myeloid lineages results in a phenotype similar to non-insulin-dependent diabetes mellitus, likely the result of alterations in tumor necrosis factor-α production (1). In vivo, the Axl receptor has been identified in vascular

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smooth muscle cells and capillary endothelial cells of synovial tissue obtained from patients with rheumatoid arthritis (34). Collectively, these results suggest that Gas 6-Axl interactions may be cell and tissue specific.

We asked whether Gas 6 regulates endothelial cell survival at growth arrest. To address this question, we characterized Axl, Rse, and Gas 6 expression in human pulmonary artery endothelial cells (HPAEC). We identified Axl and Rse expression in HPAEC and found that Axl is autophosphorylated in growth-arrested cells and exogenous Gas 6 enhances Axl phosphorylation 3.5-fold. We examined cell-associated and secreted Gas 6 expression and found that growth arrest induces Gas 6 secretion into the medium independent of cell density. Our data also demonstrate that exogenous Gas 6 promotes cellular viability but is not a growth-potentiating factor for the G protein-coupled agonist thrombin. Finally, Gas 6 inhibits programmed cell death in endothelial cells, and this response is enhanced in HPAEC that overexpress the Axl receptor. Thus the results of our studies suggest that Gas 6 functions as an antiapoptotic factor in pulmonary vascular endothelial cells through ligation with the high-affinity Axl receptor tyrosine kinase.

METHODS

Cells and culture conditions. HPAEC (Clonetics) were grown to confluence in growth medium (EBM, Clonetics) containing 1.38 μM hydrocortisone, 3 pM recombinant human epidermal growth factor (both from Clonetics), 10 μg/ml endothelial cell growth supplement (Sigma), 10,000 U/ml penicillin and streptomycin (Sigma) and supplemented with 10% fetal bovine serum (FBS, HyClone). At confluence, HPAEC were growth arrested by replacing serum-containing medium with serum-free medium supplemented with hydrocortisone, penicillin, and streptomycin and with or without recombinant human Gas 6 (Amgen), or human protein S (Enzyme Research Laboratories, South Bend, IN).

For proliferation assays, HPAEC were plated at 25×10^3 cells/cm^2 and incubated for 24 h in growth medium. Growth medium was removed, cells were washed 2× with PBS, and test medium containing 0.5% FBS supplemented with recombinant human Gas 6 (1–6 nM), human thrombin (0.1–10.0 U/ml, Enzyme Research Laboratories), or Gas 6 (1.5 nM) plus thrombin (0.1–10.0 U/ml) was added. Medium was exchanged daily, cells were trypsinized, and cell number was determined electronically (Coulter Counter, Hialeigh, FL).

PCR and Northern blot analysis. Total RNA was isolated from HPAEC and C57/Black mouse lung using the TRIzol reagent (GIBCO BRL) or the guanidinium thiocyanate method (7) and measured by optical density (260- to 280-nm absorbance ratio). RNA integrity was checked by electrophoresis through formaldehyde-agarose gels stained with 25 μM ethidium bromide. Poly(A)$^+$ RNA was purified from total RNA by oligo(dT) cellulose column chromatography (GIBCO BRL).

cDNA probes for Northern blot analysis were generated using RT-PCR with total RNA isolated from either U937 cells (for Axl sequences) or HPAEC. The primers were 5′-GCAGGCTGAAGAAAGTCCCTTCG and 3′-GCTGGCTGACCACTATCCACTC for Axl; 5′-CTGCACTGTGGAGGGATGGAG and 3′-GCCACACTGGCTGGGAGATCTCGG for Rse; 5′-CAATCTCTGTTGAGGAGCTGG and 3′-GACCACGTGCTCTTGGCCGTC for Gas 6, and 5′-CCTTCCTGGGCATGGAGTCCTG and 3′-GGAGCAATGATCTTGATCTTC for β-actin. PCR products were radiolabeled and hybridized to total RNA and poly(A)$^+$ RNA immobilized on nylon membranes (Amersham).

HPAEC metabolic labeling and Rse immunoprecipitation. Rse receptor biosynthesis was determined in HPAEC by metabolically labeling confluent cultures of cells with [^{35}S]methionine (ICN) for 4 h after a 1-h incubation in methionine-free medium (GIBCO BRL). HPAEC extracts were prepared to enrich for membrane and cytoplasmic proteins and exclude cell nuclei from the preparation. HPAEC extracts were prepared by standard techniques. Protein concentration was determined by the Bio-Rad protein assay, and equal concentrations of cellular extracts were used for immunoprecipitation. Rse was immunoprecipitated from extracts with a polyclonal antibody raised against the amino terminus of Rse and defined here as anti-Rse IgG (originally anti-Sky IgG, the generous gift of Dr. Kensako Mizuno, Kyushu University, Fukuoka, Japan) (35). Rabbit anti-Rse or normal rabbit serum immunoprecipitates were collected on protein A (A/G) agarose (Santa Cruz Biotechnology), and bound proteins, eluted with SDS buffer, were electrophoresed on 7.5% polyacrylamide gels and prepared for fluorography.

Immunoprecipitation and Western blot analysis of Axl and Gas 6. Cell lysates for cell-associated Axl and Gas 6 were prepared as described for biosynthetic labeling but without radioisotope. Gas 6 was identified in HPAEC-conditioned medium by collecting medium after 2, 4, and 5 days of serum depletion. Conditioned medium was concentrated 40-fold by centrifugation in a concentrator fitted with a YM-30 membrane (Amicon). Concentrated medium and cell lysates were prepared for electrophoresis through 10% polyacrylamide gels, transferred to nitrocellulose membranes (Schleicher and Schuell), and detected by successive incubations with anti-Gas 6 antibody (Amgen), anti-rabbit IgG labeled with horseradish peroxidase (Santa Cruz Biotechnology), and enhanced chemiluminescence (Pierce).

Western blot analysis for Axl detection was as described for Gas 6 except that the primary antibody was an affinity-purified, rabbit anti-Axl IgG (Amgen). Axl was also immunoprecipitated from cell lysates using a goat anti-Axl IgG (Santa Cruz Biotechnology). Alternatively, tyrosine-phosphorylated Axl receptor was immunoprecipitated from confluent HPAEC cultures serum deprived for 24 h and then left untreated or treated with Gas 6, protein S, or FBS for 5 min. Tyrosine-phosphorylated Axl was immunoprecipitated from cell lysates with the monoclonal anti-phosphotyrosine IgG clone 4G10 (Upstate Biotechnology). Blotted proteins were detected using either the rabbit anti-Axl IgG from Amgen or a second rabbit anti-Axl IgG (the generous gift of Dr. E. Liu, University of North Carolina, Chapel Hill, NC).

Apoptosis assays. DNA fragmentation was determined as described (16). Briefly, cells were grown to confluence in serum-containing medium, at which time serum was removed and cells were cultured for 72–120 h in serum-free medium with one medium exchange at 48 h. In all three apoptosis assays described below, each experiment included a negative and a positive control. Cells maintained in serum-containing medium were used as a negative control, and cells maintained in serum-containing medium supplemented with 1 μM staurosporine (Sigma), a protein kinase inhibitor that induces apoptosis, were used as a positive control (16). DNA was isolated from both floating cells, which were pelleted from test medium, and attached cells. The samples were electrophoresed through a 1.8% agarose gel containing 25 μM ethidium bromide.

For Hoechst staining, cells were grown to confluence on glass coverslips, serum deprived, and treated with various factors. Cells were fixed on *days 3* and *4* and stained simultaneously by inverting glass coverslips onto a drop of staining solution containing 4% formaldehyde, 0.6% Nonidet P-40, and 18.7 μM Hoechst 33258 (Sigma) in PBS at room temperature for 30 min (28). Fifty cells from three fields were counted for each condition in duplicate. Cells were scored as apoptotic if they displayed a highly condensed and fragmented nucleus.

Annexin V-positive- and propidium iodide-negative-stained HPAEC were detected by flow cytometry. HPAEC were grown as described previously except that cells were harvested on *days 2* and *3* of serum-free culture. Cells were trypsinized, counted, and costained with fluorescein-conjugated annexin V and propidium iodide (R&D Systems). Cells were analyzed by flow cytometry (Becton Dickinson) and quantitated using Cell Quest software.

Retroviral transduction of HPAEC with Axl constructs. A full-length cDNA encoding the Axl gene (gift of Dr. E. T. Liu) was subcloned into the *Eco*RI site of pMSCVpac (15). The Axl-retroviral construct was transfected into Phoenix cells to generate retroviral supernatants as previously described (6). Transduced HPAEC were selected by puromycin resistance and analyzed between *passages 6* and *9*.

Statistics. The data are expressed as means ± SD. Analysis of variance was carried out using the two-factor ANOVA. Statistical analysis comparing cells maintained in serum-free medium in the presence or absence of Gas 6 was conducted using Student's *t*-test. Differences were significant at $P < 0.05$.

RESULTS

Vascular endothelial cells express the RTK Axl and Rse. We amplified a 193-bp Axl fragment, a 208-bp Rse fragment, and a 589-bp Gas 6 fragment from HPAEC by RT-PCR. Axl and Rse expression were confirmed by Northern blot analysis of HPAEC RNA (Fig. 1*A*). Northern blotting revealed the presence of a major band migrating at 4.2 kb for Rse mRNA. The Axl probe identified a single major transcript at 5 kb and a second transcript just visible at 3.4 kb. It is noteworthy that in transformed and tumorigenic cells, both the 5.0- and 3.4-kb Axl transcripts are represented equally (33). Quantitation of the 5.0-kb Axl and 4.2-kb Rse mRNAs compared with β-actin from the same cell sample demonstrates that Rse mRNA is 2.2 times more abundant than Axl mRNA in HPAEC.

Gas 6 is expressed in pulmonary endothelial cells in culture and in whole lung. Previous investigations identified the ligand Gas 6 in many cells and organs, but particularly high levels were identified in HUVEC and bovine aortic endothelial cells and in human and murine lungs (2, 24, 37). We examined Gas 6 expression in endothelial cells isolated from human pulmonary artery and tested whether our human cDNA Gas 6 probe also hybridizes to murine Gas 6. Northern blot analysis of Gas 6 transcripts (Fig. 1*B*) revealed the presence of a single major band migrating at ~2.6 kb in HPAEC and in whole lung extracts from C57/Black mice.

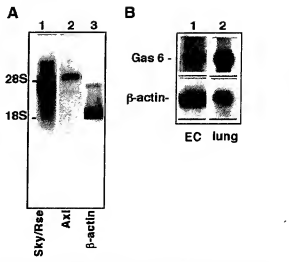

Fig. 1. Northern blot of receptor tyrosine kinases Rse and Axl and Gas 6 poly(A)$^+$ RNAs. *A*: human pulmonary artery endothelial cell (HPAEC) RNA was harvested and 2 μg of poly(A)$^+$ RNA were added to each lane, electrophoresed, and blotted to nitrocellulose. cDNA probes for Rse (*lane 1*), Axl (*lane 2*), and β-actin (*lane 3*) were successively hybridized to the same blot. The ribosomal bands are indicated as 28S and 18S. *B*: RNA was harvested, and 2 μg of poly(A)$^+$ RNA were added to each lane, electrophoresed, and blotted to nitrocellulose. cDNA probes for Gas 6 and β-actin were successively hybridized to the same blot. *Lane 1*, HPAEC RNA; *lane 2*, C57/Black mouse lung RNA. Northern blots shown are representative images from 3 independent experiments.

Immunoprecipitation and Western blot analysis of Axl and Rse. We examined cell lysates for the presence of the Axl and Rse receptors. Using three different Axl antibodies, we detected several forms of the immunoreactive Axl RTK. For example, with a rabbit polyclonal Axl antibody, the Axl receptor appears as a single major band with a relative mobility of 125 kDa and a second minor band with a relative mobility of 104 kDa (Fig. 2, *lane 1*). When Axl was immunoprecipitated using a goat polyclonal Axl antibody and then blotted with a second rabbit Axl antibody, the Axl receptor is seen as a doublet with a relative mobility of 140 and 110 kDa (Fig. 2, *lane 3*). A similar pattern was observed when cells were metabolically labeled and immunoprecipitated with these same two immunoreagents (not shown). Anti-Axl antibody premixed with a fivefold molar excess of an Axl-Fc fusion molecule failed to recognize all forms of the Axl receptor. Several forms of the Axl receptor, which correspond in relative mobility to those shown in Fig. 2, have been described in other cell types as the precursor (p104) Axl polypeptide and partial (p120) and fully glycosylated (p140) forms of Axl (32).

The Rse receptor was not detected by Western blot of HPAEC lysates; Rse was detected only by metabolically labeling cells before immunoprecipitation with an anti-Rse antibody. The Rse receptor is reported as a 140-kDa polypeptide in Rse-transfected Chinese hamster ovary cells, and this particular anti-Rse IgG also immunoprecipitates a Src kinase, a 60-kDa polypeptide (38). In HPAEC, immunoprecipitation with anti-

Rse IgG revealed the presence of three polypeptides with a relative mobility of 54, 131, and 181 kDa (Fig. 3, arrowheads).

Growth arrest induces Gas 6 secretion independent of cell density. Because Gas 6 expression is associated with growth arrest, we asked whether cell density affects Gas 6 expression and secretion. Therefore, we examined HPAEC cultures under sparse (8×10^3 cells/cm^2) and confluent (32×10^3 cells/cm^2) cell densities for cell-associated and soluble forms of Gas 6 (Fig. 4). We found that HPAEC maintained in culture under either serum-free or low-serum conditions (0.5% FBS) expressed Gas 6 regardless of cell density. Densitometric analysis from four independent experiments revealed that 1.1 ± 0.3 ng Gas 6 per 1×10^6 cells accumulates in the conditioned medium of confluent cultures. Densitometric analysis from two experiments revealed that 1.4 ± 0.1 ng Gas 6 per 1×10^6 cells accumulates in the conditioned medium of sparse cultures. The cell-associated forms of Gas 6 that were present at 2 and 4 days of serum deprivation correspond to the mature polypeptide, with a relative mobility of ~70 kDa, a higher molecular mass form at 110 kDa (probably a dimer), and a third immunoreactive species at 50 kDa (likely an intracellular precursor or degradation product) (Fig. 4). The 70-kDa form is the predominant form present in the conditioned medium of both sparse and confluent serum-deprived HPAEC at both 2 (data not shown) and 4 days of culture. The anti-Gas 6 antibody readily detects between 0.2 and 2 ng of the recombinant human Gas 6 (Fig. 4, *lanes 5–7*) but does not cross-react with 20 ng of recombinant human protein S (data not shown).

Fig. 2. Different forms of the Axl RTK are present in HPAEC. HPAEC were grown to confluence, and cell extracts were prepared, electrophoresed through a 7.5% polyacrylamide gel, and blotted. Four immunoreactive forms of the Axl RTK were detected by different anti-Axl antibodies. *Lane 1*, immunoblotting of HPAEC extracts (0.15×10^6 cells) with an anti-Axl IgG (Amgen); *lane 2*, competition with a soluble form of the Axl receptor; *lane 3*, immunoprecipitation and immunoblotting with 2 different anti-Axl antibodies (Santa Cruz Biotechnology and E. Liu). Molecular mass markers (kDa) are indicated. Images shown are representative of 3 independent experiments.

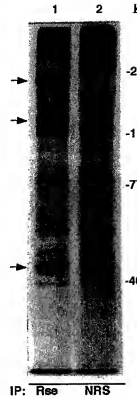

Fig. 3. Immunoprecipitation of the Rse receptor. HPAEC were grown to confluence and labeled with [^{35}S]methionine, and extracts were immunoprecipitated with anti-Rse antibody (Rse) or normal rabbit serum (NRS). The presence of 3 polypeptides specifically immunoprecipitated with anti-Rse antibody are indicated by arrowheads. Molecular mass markers (kDa) are indicated. A second independent experiment showed similar results.

Axl receptor is constitutively phosphorylated in HPAEC. The expression and secretion of Gas 6 in HPAEC led us to ask whether endogenous Gas 6 binds and activates its receptors. We found that the Axl receptor is phosphorylated in untreated cells (Fig. 5, *lane 1*). Moreover, the addition of exogenous Gas 6 (Fig. 5, *lane 2*) but not of serum (Fig. 5, *lane 3*) or protein S (data not shown) enhances Axl phosphorylation 3.5-fold. Phosphorylated Rse receptor was not detected (data not shown).

Gas 6 effects on HPAEC proliferation. In cell types that express Gas 6 plus both the Axl and Rse receptors, a mitogenic and/or antiapoptotic function for Gas 6 has been identified (10, 12, 21, 30, 31). Thus the presence of both the ligand Gas 6 and the two receptors Axl and Rse suggested that Gas 6 has proliferative and antiapoptotic properties in HPAEC. Our data show that the addition of recombinant human Gas 6 to HPAEC cultures results in a statistically significant increase in cell number (Fig. 6). The maximal increase in cell number occurred with exposure to 1.5 nM Gas 6 (100 ng/ml), resulting in a 36% increase in cell number. Higher concentrations of Gas 6, i.e., 3.0 and 6.0 nM (200 and 400 ng/ml) did not enhance the proliferative

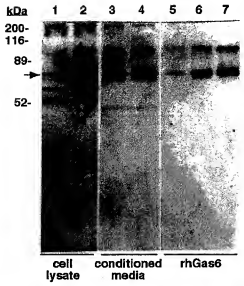

Fig. 4. Gas 6 is cell associated and released into conditioned medium. HPAEC were incubated for 4 days in serum-free medium. The immunoblot shows conditioned medium and cellular extracts after electrophoresis through a 10% polyacrylamide gel. *Lane 1*, Gas 6 detected by immunoblot of cell lysates from sparse cultures; *lane 2*, confluent cultures; *lane 3*, Gas 6 is also released into serum-free medium from sparse cultures; *lane 4*, confluent cultures; *lanes 5–7*, 0.2, 1, and 2 ng, respectively, of recombinant human Gas 6 (rhGasb). Gas 6 is indicated by arrow. Molecular mass markers (kDa) are indicated. Images shown are representative of 4 independent experiments (see text).

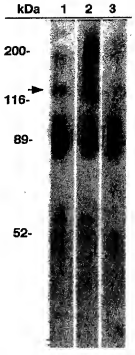

Fig. 5. Axl phosphorylation in growth-arrested HPAEC. HPAEC were grown to confluence in serum-containing growth medium. Quiescent HPAEC were either untreated or treated with Gas 6 or fetal bovine serum (FBS) for 5 min, lysed, and immunoprecipitated with the anti-phosphotyrosine antibody clone 4G10. Immunoprecipitated proteins were electrophoresed on a 7.5% polyacrylamide gel and immunoblotted with anti-Axl antibody. The Axl receptor is indicated by the arrowhead. Molecular mass markers (kDa) are shown. The results shown represent 3 independent experiments.

response further. In contrast, exposure to 10% FBS caused a 180% increase in cell number. The HPAEC response to Gas 6 stimulation is similar to previous findings by other investigators analyzing nonendothelial cell types (12, 21, 30).

It has been demonstrated previously that Gas 6 is a growth-potentiating factor for G protein-coupled receptor agonists such as thrombin and angiotensin II (21, 30). We tested thrombin alone (from 0.1 to 10.0 U/ml) and in combination with Gas 6 in our proliferation assays. However, neither thrombin alone nor in combination with 1.5 nM Gas 6 showed mitogenic or growth-potentiating activity (data not shown).

Apoptosis in HPAEC. Previous studies have identified antiapoptotic functions for Gas 6 in nonendothelial cells maintained under serum-free conditions (3, 12, 25, 31). HPAEC, like other endothelial cells, will apoptose if deprived of serum and growth factors. Therefore, we optimized the culture conditions to promote apoptosis in HPAEC before testing whether Gas 6 affects HPAEC survival.

In confluent cultures of HPAEC, DNA fragmentation is easily detected in cells maintained under serum-free culture conditions (Fig. 7, *lanes 4* and *5*) but not in cells grown in serum-containing medium (Fig. 7, *lane 3*). DNA fragmentation induced by staurosporine treatment is shown for comparison (Fig. 7, *lane 2*). These data confirm that serum-free culture conditions induce programmed cell death in HPAEC.

We used cellular morphology in conjunction with Hoechst staining to quantify attached apoptotic HPAEC and double labeling with annexin V and propidium iodide to quantify floating and attached apoptotic HPAEC. Gas 6 treatment results in a significant and reproducible survival effect on HPAEC maintained in serum-free conditions as measured by Hoechst staining and double labeling, as shown in Fig. 8. The addition of Gas 6 results in a 47% decrease in attached

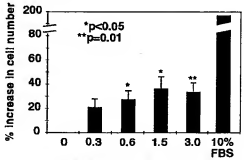

Fig. 6. Gas 6 increases HPAEC viability. HPAEC were plated at 25×10^3 cells/cm^2 and incubated for 24 h in growth medium. Growth medium was removed, and test medium containing 0.5% FBS with and without recombinant human Gas 6 (1–6 nM) was added. Cells maintained in 10% FBS were used as a control. Cell number was determined on *day 5*. The percent increase in cell number compared with untreated cells in shown. Values shown are means from 4 independent experiments.

Fig. 7. Serum depletion induces apoptosis in HPAEC. HPAEC were cultured for 4 days in the presence or absence of serum, and genomic DNA was isolated and electrophoresed on a 1% agarose gel containing ethidium bromide. *Lane 1*, DNA molecular mass marker; *lane 2*, DNA from HPAEC treated with staurosporine (positive control); *lane 3*, DNA from HPAEC grown in 10% serum (negative control), *lanes 4* and *5*, DNA from HPAEC maintained in serum-free medium. The results shown are representative of 4 independent experiments.

apoptosing HPAEC on *day 4* (Gas 6 10%, control 19%, $P < 0.001$) and a 28% decrease in floating plus attached HPAEC undergoing apoptosis on *day 2* (Gas 6 10%, control 14%, $P = 0.001$). Finally, under all the conditions shown in Fig. 8, there was no significant difference in the number of necrotic or dead cells (i.e., annexin V positive/propidium iodide positive) measured by flow cytometry (data not shown). Collectively, these results suggest that both the endogenous and exogenous Gas 6 function to inhibit HPAEC programmed cell death.

Axl mediates Gas 6 antiapoptotic function. The Axl receptor exhibits the highest affinity for Gas 6 com-

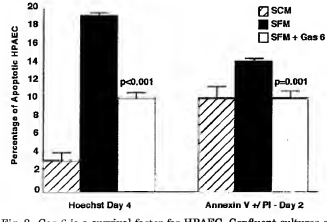

Fig. 8. Gas 6 is a survival factor for HPAEC. Confluent cultures of HPAEC were treated in either serum-containing medium (SCM, hatched bars), serum-free medium (SFM, solid bars), or serum-free medium supplemented with 1.5 nM recombinant human Gas 6 (open bars). Apoptosis was evaluated by Hoechst staining on *day 4* or by flow cytometry of annexin V-positive and propidium iodide-negative HPAEC on *day 2*. Values are the means from 3 independent experiments for Hoechst staining or 5 independent experiments for flow cytometry.

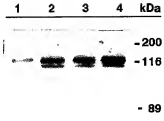

Fig. 9. Overexpression of Axl in transduced HPAEC. Nontransduced and Axl^{wt}-transduced HPAEC were grown to confluence, and cell extracts were prepared, electrophoresed through a 7.5% polyacrylamide gel, and blotted with an anti-Axl IgG (Amgen) as described. *Lanes 1* and *2*, nontransduced HPAEC extract (30 and 60 μg total protein per lane, respectively); *lanes 3* and *4*, Axl^{wt} HPAEC extract (30 and 60 μg total protein per lane, respectively). Molecular mass markers (kDa) are indicated. Images shown are representative of 3 independent experiments.

pared with Rse and Mer (29). Therefore, to test Gas 6-receptor interactions during HPAEC survival, we generated Axl-transduced HPAEC using a full-length Axl cDNA (Axl^{wt}). We quantified Axl expression in transduced and nontransduced HPAEC by Western blot analysis and found a twofold increase in ectopic Axl expression (a representative blot is shown in Fig. 9). We used the Axl^{wt} HPAEC to test the effect of Gas 6 on cellular survival. We found that Gas 6 decreases the number of apoptotic Axl^{wt} HPAEC by 54% (Gas 6 5%, control 11%, $P < 0.05$) as shown in Fig. 10.

DISCUSSION

The vascular endothelium is a monolayer of contact-inhibited, growth-arrested cells lining the luminal sur-

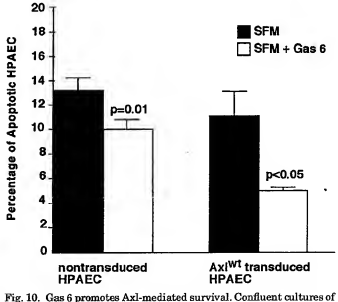

Fig. 10. Gas 6 promotes Axl-mediated survival. Confluent cultures of nontransduced HPAEC and Axl^{wt}-transduced HPAEC were treated for 2 days in serum-free medium (solid bars) or serum-free medium supplemented with 1.5 nM recombinant human Gas 6 (open bars). Apoptosis was evaluated by flow cytometry of annexin V-positive and propidium iodide-negative HPAEC. Values are the means from 4 independent experiments for flow cytometry.

face of the mature blood vessel wall. The molecular mechanisms contributing to the unique longevity of endothelial cells remain undefined. Gas 6 is a mitogen and survival factor for various cell types, transducing signals through its receptors Axl and Rse. To determine whether the Gas 6 signaling pathway is a potential mediator of endothelial cell survival at growth arrest, we examined the expression of Gas 6 and the receptors Axl and Rse and characterized the proliferative and antiapoptotic activities for Gas 6 in pulmonary endothelial cells in vitro.

We found that HPAEC simultaneously express both the Axl and Rse RTK. Axl and Rse receptors are detected from either total RNA or poly(A)$^+$ RNA, with a twofold higher steady-state level of Rse mRNA. Conversely, Western blot analysis indicates the Axl receptor is more highly expressed than the Rse receptor; this is most likely a reflection of differing antibody affinities rather than true differences in protein expression levels.

Gas 6 was originally identified as one of several molecules whose expression negatively correlates with cellular proliferation and serum depletion (24, 36). We questioned whether growth arrest by serum deprivation differs from growth arrest by contact inhibition in regard to Gas 6 expression and secretion. Measurement of Gas 6 levels in sparse vs. confluent cell cultures under serum-free conditions demonstrated no significant difference in cell-associated or soluble Gas 6 between the two cell densities, indicating that serum deprivation induces Gas 6 expression in vitro and contact-inhibited growth does not further augment Gas 6 expression. These results are in contrast to a recent study in which soluble Gas 6 was detected in the cell-associated fraction but not in the conditioned medium of HUVEC, suggesting that secreted Gas 6 may be completely bound to cell surface receptors (2). The difference between our findings and those of Avanzi et al. (2) may be due to the detection assays (i.e., Western blot vs. ELISA, respectively) or to the heterogeneity of endothelial cells isolated from different vascular beds. However, our data confirm a previous report demonstrating that Gas 6 is released into the conditioned medium from bovine aortic endothelial cells (37). Our results demonstrate that HPAEC growth arrested by either contact inhibition or serum depletion secrete Gas 6, which remains in a soluble form in the conditioned medium.

Previous studies revealed that Gas 6 is a growth-potentiating factor for the G protein-coupled receptor agonists such as thrombin and angiotensin II (21, 27, 30). Furthermore, it was shown that Gas 6 mitogenic activity is separable from Gas 6 antiapoptotic function; Gas 6 induces entry into the S phase of the cell cycle in the presence of low serum but is an antiapoptotic factor in the complete absence of serum (and growth factors) (3, 12). We found that at sparse cell densities in low-serum-containing medium, there is a statistically significant increase in HPAEC cell number in the presence of increasing concentrations of exogenous Gas 6. However, this proliferative response observed after 5 days of Gas 6 treatment may represent increased cell viability and not entry into S phase. The small increase in cell number (~5–7% per day) makes it difficult to test this hypothesis by standard techniques (e.g., measurement of [^{3}H]thymidine incorporation or 5-bromo-2′-deoxyuridine). In addition, we were unable to detect a Gas 6 growth-potentiating effect in the presence of thrombin. This finding supports the results of a previous study demonstrating that thrombin has a differential effect on endothelial cells isolated from distinct vascular beds and that long exposures to thrombin inhibit endothelial cell mitogenesis regardless of endothelial cell type (39). Our findings support the supposition that Gas 6 increases cell viability rather than stimulating mitosis in HPAEC.

Our data demonstrate that Gas 6 has an antiapoptotic function for HPAEC. Although the total population of HPAEC undergoing apoptosis on *day 2* (or *day 3*) of serum-free culture is relatively small (14% of total cells), the small number of apoptotic endothelial cells is in agreement with studies conducted on NIH/3T3 cells in which Gas 6 treatment decreased the number of apoptotic cells from ~11 to 4% (3). Hoechst staining revealed similar numbers of apoptosing HPAEC on *days 3* and *4* of serum-free culture. Furthermore, overexpression of the full-length Axl cDNA results in over a twofold increase in Axl protein levels and a corresponding decrease in the percentage of apoptotic cells. The results of studies examining apoptosis in the vessel wall in atherosclerotic lesions and regions of restenosis show a similar percentage of apoptotic cells, 2–30%, as detected by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) and Hoechst staining (11, 14, 17, 23). Moreover, in a single study addressing tumor angiogenesis, the complete removal of vascular endothelial growth factor resulted in the detachment of endothelial cells and subsequent tumor regression; however, only occasional TUNEL-positive endothelial cells could be identified within the blood vessel wall at any single time point (4). Thus the low numbers of HPAEC undergoing apoptosis in our studies are consistent with the results of studies performed on whole vessels. Collectively, our functional studies reveal that Gas 6 causes an increase in viability and a decrease in apoptosis, suggesting that Gas 6 is a survival factor for HPAEC.

Our studies indicate that the Axl receptor is constitutively phosphorylated and the addition of exogenous Gas 6, but not of serum or protein S, increases Axl phosphorylation 3.5-fold. These data indicate that Axl phosphorylation occurs via Gas 6 ligation. We also detected a 54-kDa protein that coprecipitates with the metabolically labeled Rse, which may be a member of the Src family of kinases (Fig. 3) (38), and a higher molecular mass band that may be a Rse-Src complex or a Rse-Gas 6 complex. These results support the supposition that Gas 6 promotes HPAEC survival through constitutive ligation with Axl and/or the Rse RTK.

It remains unknown whether Gas 6 interacts with both receptors or whether Axl and Rse can form heterodimers following ligand binding. The cell types

identified in which Gas 6 is a growth-potentiating and a survival factor express one or both receptors (Axl and Rse) in addition to the ligand (Gas 6) (12, 25, 31). These data support the hypothesis that the complex biology of the Gas 6 signaling pathway is regulated by cell type-specific expression of the Gas 6 receptors.

Our measurements indicate that picomolar concentrations of Gas 6 are synthesized by HPAEC under serum-free conditions. However, nanomolar concentrations are required for a cellular response in vitro, both in our studies and in independent studies of several cell types (3, 10, 12, 21, 25, 29). There are at least two possibilities that could explain this difference. Endogenous Gas 6-Axl interactions may not promote HPAEC survival. We think this is unlikely because gene deletion studies indicate that Axl-deficient embryonic fibroblasts are more susceptible to apoptosis after serum withdrawal and are refractory to exogenous Gas 6 treatment compared with Axl wild-type embryonic fibroblasts (3). Moreover, mice null mutant for all three Gas 6 receptors display increased TUNEL-positive cells in the vessel wall (22). We favor the supposition that the amount of endogenous Gas 6 may be limiting under our defined experimental conditions, and, therefore, endogenous Gas 6 cannot completely protect from apoptosis after serum withdrawal. This scenario would explain why we do not observe an increased cell survival in the Axl^{wt} HPAEC on serum withdrawal but do observe a twofold increase in survival after addition of exogenous Gas 6.

Programmed cell death is an integral component of the vascular response to injury. On the one hand, apoptosis in vascular smooth muscle cells counters the exuberant cellular proliferation that leads to intimal thickening (8, 18). On the other hand, apoptosis in vascular endothelium contributes to pathogenesis by promoting intravascular coagulation activation (5). Apoptosis also has a role in the vascular remodeling associated with tumor angiogenesis (4). Thus a balance between cell growth and cell death may be required for vascular remodeling. In this report, we characterized the expression and function of the Gas 6 signaling pathway in pulmonary endothelium in vitro. Further elucidation of this pathway will reveal whether Gas 6 functions in maintaining the equilibrium between cell growth and survival in lung endothelium in vivo.

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